

# Mode of Action of an Inhibitor from Agar on Growth and Hemagglutination of Group A Arboviruses

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## Mode of Action of an Inhibitor from Agar on Growth and Hemagglutination of Group A Arboviruses

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### ABSTRACT

COLÓN, JULIO I. (Fort Detrick, Frederick, Md.), JANE B. IDOINE, ORVILLE M. BRAND, AND RICHARD D. COSTLOW. Mode of action of an inhibitor from agar on growth and hemagglutination of group A arboviruses. *J. Bacteriol.* 90:172-179, 1965.—A polysaccharide obtained from agar, and having properties similar to a previously described sulfated polysaccharide, was observed to inhibit growth and hemagglutination of some group A arboviruses. The evidence presented confirms that the inhibitory activity, in part, is the result of direct interaction between the agar polysaccharide (AP) and free virus particles. Additional evidence indicates that inhibition of viral growth also occurs as the result of interaction between AP and the chick-fibroblast cells used for propagation of the virus. The possibility was considered, therefore, that at least two different inhibitors could be present in AP—one that reacts directly with the virus particle and another that reacts with host cells. AP does not induce the production of interferon in the test system used.

Takemoto and Liebhaber (1961) showed that a sulfated polysaccharide present in agar inhibited the growth of minute plaque-forming strains of encephalomyocarditis (EMC) virus. They also showed that this substance interfered with the agglutination of sheep red blood cells by the same EMC strains. Further reports by these authors described similar inhibition of EMC and other viruses by polysaccharides other than those extracted from agar, such as sodium dextran sulfate (Takemoto and Liebhaber, 1962; Liebhaber and Takemoto, 1963). Other investigators (Schulze and Schlesinger, 1963; Colón, Idoine, and Brand, 1963) have described inhibitors extracted from agar which interfere with viral growth and the agglutination of red blood cells by certain arboviruses. Most of the published data on inhibitors obtained from agar show that their inhibitory effect was due, at least in part, to direct interaction with the virus particles. The inhibitory effects of these sulfated polysaccharides from agar have also been reversed or prevented by polyelectrolytic substances such as diethylaminoethyl (DEAE) dextran (Liebhaber and Takemoto, 1961; Ushijima et al., 1962). This direct mode of action of the agar inhibitor on the virus was generally confirmed and extended for the group A arboviruses used in this study. In addition,

results were obtained that suggest a mode of action based on the interaction between the inhibitor and chick-fibroblast cells.

### MATERIALS AND METHODS

**Viruses.** Eastern equine encephalitis (EEE) virus (CDC strain SC 7), received as a 20% chick-embryo suspension from the Communicable Disease Center, Montgomery, Ala., was used after two passages in chick embryos in this laboratory. Other viruses used were Venezuelan equine encephalitis (VEE) virus, Trinidad strain (Hardy, 1950), attenuated VEE virus, 9t (Hearn, 1961), and western equine encephalitis (WEE) virus, Rockefeller strain, obtained from U.S. Army Medical School as a 10% suspension of fourth mouse brain passage.

**DEAE dextran.** DEAE dextran was obtained from Pharmacia Laboratories, Uppsala, Sweden. The preparation used in these experiments had been prepared from dextran with a molecular weight of approximately  $2 \times 10^6$ . A 1% stock solution of DEAE dextran in water was autoclaved for 10 min at 121 C and stored at 4 C.

**Sodium dextran sulfate (NaDS).** NaDS was also obtained from Pharmacia Laboratories, Uppsala, Sweden. The preparation used in these experiments was NaDS 2000, which contained 15 to 18% sulfur and had been prepared from dextran with a molecular weight of approximately  $2 \times 10^6$ .

**Preparation of extract from agar.** The extract was prepared by the method described by Takemoto and Liebhaber (1961), modified only by eliminating a final ethyl alcohol precipitation after

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dialysis. The final preparation was dried in a rotary flash evaporator at 40 C. Stock suspensions of the dried material were prepared in sterile water and kept at 4 C or frozen at -70 C. The final product had properties similar to those described by Takemoto and Liebhaber (1961) for the sulfated agar polysaccharide and is hereafter referred to as AP (agar polysaccharide).

**Assay of virus infectivity.** Infectivity of virus samples was determined by plaque formation in primary chick-fibroblast (CF) monolayers grown in 60-mm plastic petri dishes. The virus, appropriately diluted in beef heart infusion broth (BHIB), was pipetted in 0.1-ml volumes onto the monolayer cultures that had been washed once with saline A (Puck, Cieciura, and Fisher, 1957). After 20-min adsorption at 25 C, the excess inoculum was removed and the monolayers were overlaid with nutrient agar. Plates were incubated at 37 C in a humidified atmosphere of 96% air and 4% CO<sub>2</sub>. The details of the procedure have been described by Colón and Idoine (1964).

Unless otherwise described, when assays were performed on cultures that had been treated with AP or NaDS, the monolayers were washed two times with saline A before being inoculated with virus and then were washed once again after removal of excess inoculum.

**Preparation of lactalbumin hydrolysate growth medium.** Growth medium for CF monolayers consisted of 0.5% lactalbumin hydrolysate, 10% calf serum, 0.075% sodium bicarbonate, and 0.002% phenol red in Hanks' balanced salt solution (BSS). The medium was sterilized by filtration through an asbestos pad in a Seitz apparatus. Antibiotics were added to the medium before use to give a final concentration of 0.250 mg/ml of dihydrostreptomycin sulfate and 125 units per ml of sodium penicillin G.

**Viral growth experiments.** Experiments on the effect of AP on viral growth were performed on CF monolayer cultures such as those used for assay of infectivity. After excess inoculum had been removed, the monolayers were washed once with saline A, and each culture received 5 ml of lactalbumin hydrolysate growth medium containing the given concentrations of AP. Viral growth was determined by plaque assay of samples of the supernatant growth medium, each sample consisting of the supernatant fluid from one culture.

**Procedure for inactivation studies.** Stock preparations of virus originating from chick embryo or tissue culture were suspended in BHIB to a concentration of approximately 10<sup>7</sup> plaque-forming units (PFU) per ml and then diluted 10-fold in the appropriate aqueous solutions of AP with a final pH of 7.4. After timed intervals of incubation, samples were diluted in BHIB and assayed immediately for infectivity.

**Hemagglutination tests.** Hemagglutination tests were conducted in microplates, by use of serological procedures similar to those described by Clarke and Casals (1958). The viral antigens were prepared by acetone-ether extraction of infected suckling mouse brain. Twofold dilutions of the

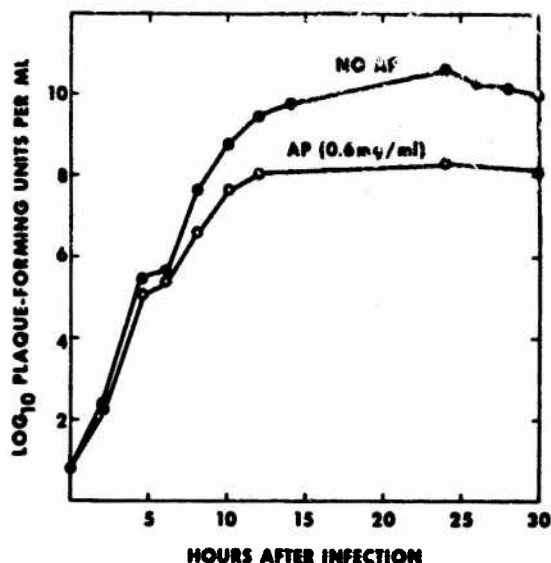


FIG. 1. Effect of sulfated agar polysaccharide (AP) on growth of WEE virus in CF monolayers. Titers obtained by assay of supernatant growth medium at indicated times after infection. Virus adsorbed to monolayers before addition of AP.

virus prepared in borate saline (pH 9.0) containing 0.1% human albumin were used to determine the titer. The indicator system of 0.14% goose red blood cells in phosphate-buffered saline was added in a volume equal to that of the diluted antigen; the test material, at a final pH of 6.35, was incubated at 37 C until the cells settled.

## RESULTS

**Effect of AP on viral growth.** Monolayers of CF cells inoculated with WEE virus at a multiplicity of 0.0001 were incubated at 37 C under growth medium containing 0.6 mg of AP per ml. At selected intervals, the supernatant fluid was assayed for content of virus (Fig. 1). Only a small difference between cultures with and without AP was observed in the amount of WEE virus released during the first cycle of growth. However, a marked inhibition of further virus production was observed in the monolayers with the medium containing AP. Thus, it appeared that, after the virus was inside a cell, the first cycle of growth was not inhibited. Similar results were obtained when the effect of AP on growth of EEE virus was studied in the same system. The infectivity of the intracellular virus in the EEE-infected cultures was examined also by assaying homogenates of washed monolayers, and it was found to be very similar whether or not AP had been present in the cultures. For example, supernatant samples of AP-free and AP-containing cultures at 4 hr after inoculation with EEE virus contained 10<sup>4.5</sup> and 10<sup>4.5</sup> PFU/ml,

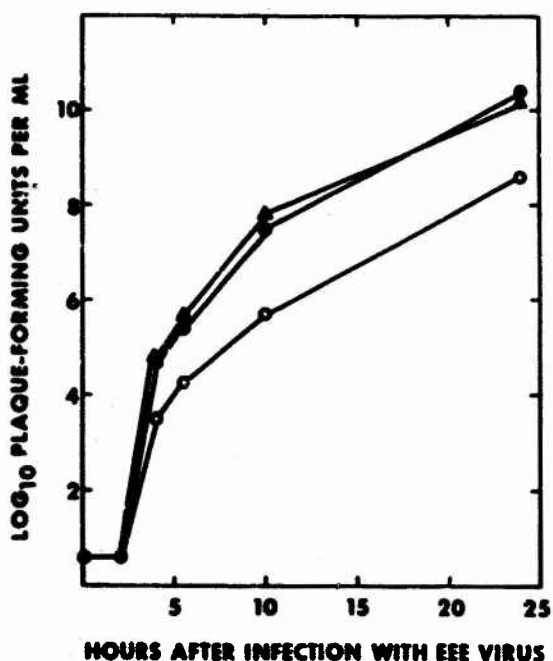


FIG. 2. Antagonistic effect of DEAE dextran on EEE virus inhibition by sulfated agar polysaccharide (AP). Virus was adsorbed to CF monolayers prior to addition of AP. Titers obtained by assay of supernatant growth medium. Growth medium contained: ●, no additive or DEAE dextran (0.1 mg/ml) alone; ○, AP (0.4 mg/ml) alone; or △, AP (0.4 mg/ml) and DEAE dextran (0.1 mg/ml).

respectively, but the corresponding intracellular titers were both  $10^{2.4}$  PFU per ml. After 24 hr, supernatant fluids contained  $10^{2.9}$  and  $10^{2.9}$  PFU/ml, respectively; the corresponding intracellular titers were  $10^{2.7}$  and  $10^{2.2}$  PFU/ml.

**Prevention of growth inhibition with DEAE dextran.** The effect of DEAE dextran on the inhibitory activity of AP was studied in monolayer cultures infected with EEE virus at a multiplicity of 0.00005. The infected cultures were incubated for various lengths of time with growth medium containing AP (0.4 mg/ml) or AP plus DEAE dextran (0.1 mg/ml). Figure 2 shows virus content of supernatant fluid from the cultures as determined by plaque titrations. The amount of DEAE dextran used completely prevented inhibition by AP. The curves for controls, containing DEAE dextran alone or with neither DEAE dextran nor inhibitor, were identical, and only one is shown. Similar experiments showed that protamine sulfate (salmine) also prevented inhibition by AP.

**Effect of AP on free virus.** The effect of AP on free virus was determined by incubating EEE or VEE virus in various concentrations of AP at 37 C. Samples were assayed by plaque titra-

TABLE 1. Reduction in plaque-forming titer of EEE virus treated with AP\* at 37 C†

Concn of AP mg/ml	Time incubated at 37 C	
	0 min	60 min
0.0	$10^{6.7}$ †	$10^{6.4}$
0.5	$10^{6.6}$	$10^{4.9}$
1.0	$10^{6.6}$	$10^{2.7}$
2.5	$10^{6.4}$	$10^{2.7}$

\* AP = sulfated agar polysaccharide inhibitor.

† Tissue culture EEE virus diluted  $10^{-2}$  in beef heart infusion broth (BHIB) was mixed 1:10 with the desired concentration of inhibitor, incubated at 37 C, and diluted further in BHIB at the indicated times.

‡ Results are expressed as plaque-forming units per milliliter.

TABLE 2. Effect of pH\* on AP-virus interaction

Buffer	pH	Per cent inhibition
Mellivaine.....	6.4	89
Mellivaine.....	6.9	92
Mellivaine.....	7.3	90
Mellivaine.....	7.6	89
Mellivaine.....	8.0	67
Borate.....	8.4	80
Borate.....	9.0	65

\* EEE virus was incubated with 0.73 mg/ml of AP at the indicated pH, diluted in beef heart infusion broth, and assayed on CF monolayers. Controls in buffers at various pH values without AP were negative for inhibition.

tion at zero time and after 1 hr at 37 C. Table 1 shows the results of a typical experiment with EEE virus. Incubation of EEE virus with AP resulted in a 2-log decrease in titer in 1 hr with the lowest concentration of AP tested (0.5 mg/ml). At higher concentrations of AP (1.0 to 2.5 mg/ml), 99.99% reduction of PFU was observed with EEE virus. There was no significant reduction in titer at zero time with the concentrations of AP tested. Similar experiments with VEE (Trinidad) virus revealed that no significant inactivation resulted after 1 hr of incubation at 37 C with 2.5 mg/ml of AP. The VEE virus strain (9t) was, however, inactivated by AP.

**Effect of pH on AP-virus interaction.** A suspension of EEE virus in BHIB ( $10^7$  PFU/ml) was diluted 1:10 in Mellivaine or borate buffers of the desired pH containing 0.73 mg/ml of AP. The samples were incubated for 1 hr at 37 C, and then were diluted immediately in BHIB and assayed in CF monolayers. The data in Table 2

show that interaction between the virus and AP occurred over a wide range of pH.

*Effect of temperature on AP-virus interaction.* EEE virus was incubated in the presence of AP

TABLE 3. *Effect of temperature\* on AP virus interaction*

Temp	Per cent inhibition of plaque formation
C	
0.0	79
7.5	84
15.0	76
30.0	72
37.0	95
42.0	99

\* EEE virus was incubated with 0.73 mg/ml of AP at the indicated temperature for 1 hr, diluted in beef heart infusion broth, and assayed on CF monolayers. Samples without AP were used as uninhibited controls for each temperature.

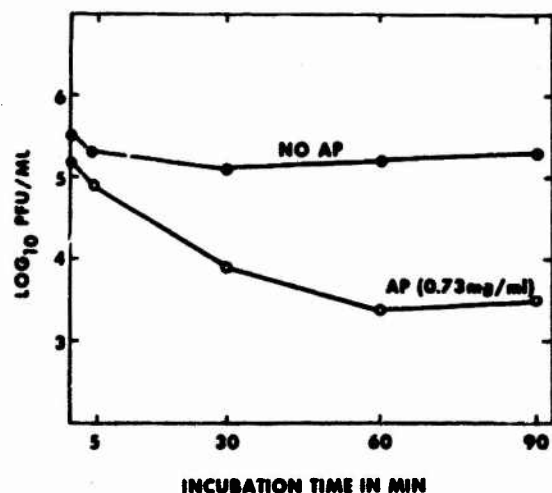


FIG. 3. *Influence of time on EEE virus-sulfated agar polysaccharide (AP) interaction at 37 C. At the designated times, samples were diluted and assayed immediately for remaining infectious units.*

(0.73 mg/ml) at various temperatures. The results (Table 3) indicated that the optimal temperature for the AP-virus interaction that resulted in decreased plaque titers was 42 C. Higher temperatures than those shown were deleterious to virus infectivity; i.e., control samples in the absence of AP were not decreased until the temperature of incubation exceeded 42 C.

*Effect of time on AP-virus interaction.* EEE virus was incubated at 37 C in the presence of AP (0.73 mg/ml). At various time intervals, samples were diluted in BHIB and assayed at once. Figure 3 shows that incubation of EEE virus with the AP at 37 C brought about a reduction in titer that increased with time up to 1 hr. In other experiments, further reductions in titer were observed up to 3 hr of incubation. No significant reversal of inhibition was observed upon dilution of the samples in BHIB.

*Other factors affecting AP-virus interaction.* The inhibitory activity of AP was antagonized by added protein or by phosphate. The data in Table 4 show that when phosphate buffer or bovine plasma albumin (BPA) was present, there was little or no inhibitory effect by AP. The inhibition by AP was greatest when the BPA concentration was the lowest. In these experiments, pH was maintained at 7.6 with Michaelis buffer, which had no adverse effect on the virus or the inhibitory activity of AP. The addition of calf serum in place of BPA had the same effect as BPA in antagonizing the inhibitory effect of AP. It is important to note that, when serum, BPA, or phosphate was present, the inhibitory effect of AP could be reversed, in part, by dilution. Thus, AP-virus interaction appears to be partly reversible in the presence of protein. It is probable that the lack of reversibility of inhibition by AP upon dilution is due to the lower concentration of protein present in the 10% BHIB used in our experiments.

*Effect of AP on hemagglutination.* The hemag-

TABLE 4. *Effect of added protein or phosphate on AP-virus interaction*

Incubation medium	Titer (PFU/ml)	
	AP absent	AP present*
BHIB (10%) in Michaelis buffer†	10 <sup>5.5</sup>	10 <sup>5.5</sup>
BPA ‡ (1%) in Michaelis buffer	10 <sup>5.5</sup>	10 <sup>5.5</sup>
BPA (0.5%) in Michaelis buffer	10 <sup>5.5</sup>	10 <sup>5.5</sup>
BPA (0.1%) in Michaelis buffer	10 <sup>5.5</sup>	10 <sup>5.5</sup>
PO <sub>4</sub> , 0.02 M	10 <sup>5.5</sup>	10 <sup>5.5</sup>

\* All samples taken after 1 hr at 37 C.

† Michaelis buffer at pH 7.6.

‡ BPA = bovine plasma albumin.



glutination reaction between equine encephalitis viruses and goose red blood cells involves specific attachment of the virus to the red blood cells (Chanock and Sabin, 1953, 1954). To test the effect of AP on the hemagglutination reaction of this group of viruses, goose red blood cells were suspended in various concentrations of AP prior to use in the test. The data in Fig. 4 show that hemagglutination was inhibited by AP least with VEE virus, more with WEE virus, and most with EEE virus, which is similar to the sequence observed for growth and plaque formation. The inhibitory effect of AP on hemagglutination was

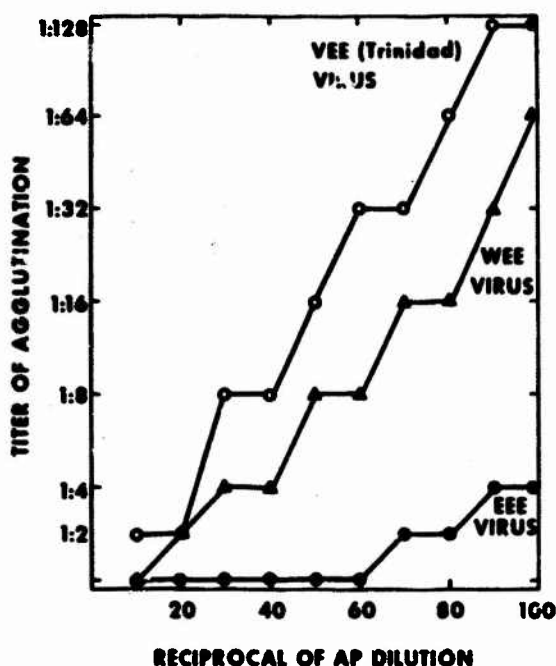


FIG. 4. Effect of sulfated agar polysaccharide (AP) on hemagglutination by three equine encephalitis viruses. AP was added to the red blood cell suspension prior to addition of antigen. Details of test conditions are described in text.

greater if the antigen was incubated with AP at 37 C for 90 min prior to the addition of goose red blood cells than if the AP was incubated first with the red blood cells. An interesting observation was that DEAE dextran mixed with AP agglutinated the goose red blood cells in the absence of antigen, but cells were not agglutinated by either AP or DEAE dextran alone.

**Interaction between AP and CF cells.** Groups of monolayer cultures were treated with AP (0.6 mg/ml) in growth medium for 1 hr at 37 C. One group of monolayers was then washed three times with saline A; another, twice with DEAE dextran (0.10 mg/ml) and once with saline A; and a third group, twice with protamine sulfate (salmine, 0.10 mg/ml) and once with saline A. After infection with EEE virus, the monolayers were washed once more with saline A and incubated at 37 C with 5 ml of growth medium. Suppression of growth where AP had been used was clearly evident from the lower titers obtained from supernatant fluid at 6 and 10 hr after virus infection (Table 5). Where AP was used, the intracellular virus titers were also significantly lower (10 and 40% of control titers at 6 and 10 hr, respectively), indicating that adsorption or penetration, or both, by the virus had been inhibited.

To establish the inhibition of EEE virus adsorption by the AP, monolayer cultures were treated with different concentrations of AP in growth medium for 1 hr at 37 C, washed three times with saline A, and inoculated with a known number of PFU of EEE virus. Table 6 shows data from a typical experiment where plaque numbers tended to decrease as the concentration of AP increased. In other experiments, monolayers treated with AP and then washed 10 times with saline A before inoculation with EEE virus still demonstrated significantly fewer plaques than cultures that received the same treatment with saline A but no AP.

**Effect of incubation time on AP-cell interaction.**

TABLE 5. Adsorption of AP\* to CF cells in monolayers and its effect on EEE virus growth

Monolayer	Time after inoculation	
	6 hr	10 hr
Control (untreated).....	$1.1 \times 10^4$ †	$1.5 \times 10^7$
AP-treated‡		
Washed with saline A.....	$1.3 \times 10^4$	$1.4 \times 10^6$
Washed with DEAE dextran.....	$6.3 \times 10^4$	$4.0 \times 10^6$
Washed with protamine sulfate (salmine).....	$2.8 \times 10^4$	$5.2 \times 10^6$

\* AP = sulfated agar polysaccharide inhibitor.

† Results are expressed as plaque-forming units in culture supernatant fluid.

‡ Monolayers were incubated with growth medium containing 0.6 mg of AP per ml at 37 C for 1 hr. Inoculum,  $10^5$  PFU of EEE virus per monolayer. At 24 hr, the titer was the same in control and experimental samples.

TABLE 6. *Inhibition of adsorption of EEE virus on chick-fibroblast monolayers by AP\**

Concn of AP	Plaque count (avg of 12 plates)
mg/ml	
None	61.0
0.175	33.2
0.350	27.1
0.700	14.4
1.400	13.5

\* Monolayers were incubated for 1 hr at 37 C with 2.5 ml of lactalbumin hydrolysate growth medium containing the given concentration of AP. Before inoculation with the virus, growth medium was removed and monolayers were washed twice with saline A.

TABLE 7. *Effect of time\* on AP-cell interaction*

Treatment time	Avg PFU per plate	
	Control	AP-treated
min		
0†	134	42
5		46
17		43
33		50
90	147	42

\* CF monolayers were treated with AP (0.70 mg/ml in 2.5 ml of lactalbumin hydrolysate growth medium), washed twice with saline A, and inoculated with EEE virus.

† AP was removed immediately after being applied to the monolayers. The operation required less than 30 sec.

Monolayer cultures were treated with 2 ml of growth medium containing 0.70 mg/ml of AP at 37 C. At intervals, duplicate plates of treated cultures were used to assay EEE virus as described in Materials and Methods. The data in Table 7 demonstrate that the interaction between AP and the cells occurred immediately, because the virus inhibition observed at zero time (<30 sec) was the same as that observed when the cells were treated for 90 min.

*Effect of pH on AP-cell interaction.* Monolayer cultures were treated for 2 min with AP (0.48 mg/ml) contained in Michaelis buffers ranging from pH 6.5 to 9.0. Michaelis buffer was used to rule out effects of different ionic strengths or qualitative differences in ions. After AP treatment, the monolayers were washed once with the corresponding Michaelis buffer. EEE virus inoculated onto the AP-treated cultures formed only 50 to 60% as many plaques as were obtained on control plates (washed with corresponding buffers) regardless of the pH conditions for the AP treatment. This observation indicates that the AP-cell interaction was independent of pH.

TABLE 8. *Inhibition of growth of equine encephalitis viruses by sodium dextran sulfate (NaDS)*

Virus	Titer of supernatant growth medium (PFU/ml)	
	Control*	NaDS†
EEE.....	10 <sup>5.3</sup>	10 <sup>4.3</sup>
WEE.....	10 <sup>5.4</sup>	10 <sup>4.7</sup>
VEE 9t.....	10 <sup>5.3</sup>	10 <sup>4.3</sup>
VEE Trinidad.....	10 <sup>5.4</sup>	10 <sup>5.2</sup>

\* Infected chick-fibroblast (CF) monolayers were incubated for 18 hr (24 hr for the EEE samples) with lactalbumin hydrolysate growth medium. After incubation, the supernatant fluid was removed and assayed on CF monolayers.

† NaDS samples were treated as controls except that 0.20 mg/ml of NaDS was contained in the growth medium.

*Effect of NaDS on infectivity of virus.* Attempts by other workers to characterize the inhibitory activity of a sulfated polysaccharide from agar on other virus groups by analogy with inhibition by other sulfated polysaccharides (Liebhaber and Takemoto, 1963) led us to study the inhibitory activity of NaDS on the equine encephalitis viruses in the systems that had been investigated with AP. To study the effect on growth, CF monolayer cultures were infected with 10<sup>5</sup> PFU of EEE virus and incubated with growth medium containing 0.20 mg/ml of NaDS. Yields of virus in supernatant fluid at 24 hr were 98% less in the presence of NaDS than those produced in control cultures. Results of this and related experiments (Table 8) show similar inhibition of WEE and VEE (9t; AP-sensitive) but no significant reduction of growth for VEE (Trinidad; AP-resistant) virus.

*Effect of NaDS on free virus.* EEE or VEE virus was incubated at 37 C in aqueous solutions containing 1.0 mg/ml of NaDS. At zero time of incubation, no inhibition was observed with either virus by plaque assay. After 60 min, the samples containing EEE virus showed 90 to 99% inhibition of infectivity in the presence of NaDS, but no inhibition was observed in samples of VEE (Trinidad) virus. NaDS caused reduction of EEE virus titer during 3.5 hr of incubation; significant inhibition of this virus was observed with as little as 1 hr of incubation with 0.016 mg/ml of NaDS. No significant reversal of inhibition on dilution of samples in BHIB was observed.

*Effect of NaDS on cells of CF monolayers.* Monolayer cultures were treated for 2 min with 0.20 mg/ml of NaDS (2 ml per plate) and then washed twice with saline A. Cultures were then used for plaque assay of two dilutions each of

EEE, VEE Trinidad, or VEE 9t virus. Treated and untreated monolayers showed no significant difference in plaque count or size when compared with plaques of control cultures for any of the viruses studied. This was interpreted to mean that, although NaDS had inhibiting activity analogous to AP, the effect on the cells was different in that NaDS was easily washed off the cells whereas AP was not (see Table 5).

#### DISCUSSION

The findings reported here have shown that inhibition of the equine encephalitis viruses by an aqueous extract from agar (AP) is similar to that reported for dengue-2 virus (Schulze and Schlesinger, 1963) and for EMC virus (Takemoto and Liebhaber, 1961). However, the following observations on AP were somewhat different from those reported by others. (i) Virus infectivity decreased with time of incubation with AP; (ii) the interaction between virus and AP was not significantly reversed on dilution of samples of virus-AP; and (iii) AP interacted with cells of CF monolayers, resulting in reduced plaque counts. On the other hand, the chemical and physical properties of the agar extract (AP) used in the studies reported here were similar to those described by others. AP was water-soluble, non-dialyzable, and contained no reducing sugars or free sulfate. Upon acid hydrolysis, AP released free sulfates and reducing sugar and lost its inhibitory properties.

The experiments described here suggested that the inhibitory effect of AP on the hemagglutination and on the growth of some members of the equine encephalitis viruses was, in part, due to direct combination of AP with the virus particles. This was shown in experiments in which plaque numbers were reduced if virus had been preincubated with AP at 37 C. AP-virus interaction was also shown by hemagglutination-inhibition experiments with goose red blood cells. When AP was incubated with the antigen prior to the test, inhibition increased with the time of incubation.

Evidence was presented that AP does interact with the cells of CF monolayers and causes inhibition of virus adsorption and perhaps penetration. With some members of the equine encephalitis viruses (WEE, EEE, and VEE 9t, but not VEE Trinidad), this was clearly shown in experiments in which the cells were treated with AP prior to viral infection. Treatment of the monolayers with AP before inoculation with virus resulted in significant reduction of plaque formation and also of early viral growth in cultures under fluid growth medium. This inhibi-

tion was apparent when the treated monolayers were washed as often as 10 times with saline A before the virus was introduced. The extent of inhibition in this case cannot be explained without assuming that AP interacts with the cells, since the amount of AP remaining nonspecifically after this dilution by washing must be insignificant. It is interesting to postulate that the inhibition observed in the AP-treated host cells may be due to interaction between AP and cell receptors for viral adsorption, or to the induction of interferon production in the cells. The latter mode of inhibitory activity has been described for statolon, a polyanionic polysaccharide (Kleinschmidt, Cline, and Murphy, 1964). In numerous experiments with AP- and NaDS-treated cultures, we have been unable to show that interferon was produced. The possibility has not been ruled out that the inoculated virus interacts with the cell-bound AP and that the inhibition is the result of AP-virus interaction.

Direct interaction of NaDS with particles of AP-sensitive equine encephalitis virus strains was shown to be similar to that observed with AP and virus. The similarity between AP and NaDS with respect to the effect on virus was demonstrated in experiments in which plaque formation was inhibited when virus had been incubated with either polysaccharide for various lengths of time at 37 C. With respect to the effect on CF cells, monolayers treated with NaDS and then washed only two times with saline A before inoculation with virus showed no effect on the AP- or NaDS-sensitive viruses studied, but monolayers treated with AP retained the inhibitory effect of AP even after 10 washings. The possibility has been considered that at least two different polysaccharides could be present in the AP preparation—one that interacts with virus particles and another that reacts with the host cells. Although AP has been separated into two fractions by paper chromatography, inhibitory activity with either fraction has not been demonstrated.

The data reported here support the conclusions of Liebhaber and Takemoto (1963) that the inhibitory effect of certain sulfated polysaccharides is related to interaction of these polyanions with oppositely charged sites on the AP-sensitive viruses, and that differences in the inhibitory response by various strains of virus may be related to differences in the surface of the respective viruses. Results demonstrating that AP-treated cells produced fewer plaques than untreated cells and that intracellular virus production proceeded normally when AP was introduced after adsorption support the proposal by



Nahmias, Kubrick, and Bernfeld (1964) that the inhibition of virus by sulfated compounds is effective at the stage of virus-to-cell adsorption.

In the case of dengue-2 virus and AP (Schulze, 1964) and of EMC virus and NaDS (Liebhauer and Takemoto, 1963), the reduced infectivity of the virus upon incubation with polysaccharide did not increase with time of incubation. Furthermore, the inhibition they observed was found to be reversible on dilution of the virus-polysaccharide samples. In the experiments reported here with EEE virus and AP, the reduction in infectivity increased with incubation time up to 3 hr, and no significant reversibility of the inhibition was observed on dilution of samples, unless the incubations were performed in the presence of 0.02 M PO<sub>4</sub> or added protein. The differences between AP and the inhibitors described by others, in respect to reversibility and the rate of the virus-polysaccharide interaction, may possibly be explained by differences in the suspension medium used for incubation.

Of some importance are the facts that certain of the equine encephalitis viruses (EEE, WEE, and VEE 9t) were markedly inhibited by polyanions by a mechanism similar to that affecting dengue-2, EMC (r+), and other viruses, and that VEE Trinidad was resistant to the inhibition. These observations should be useful in characterizing both the nature of these viruses and the mechanisms of virus-cell adsorption in infection.

#### LITERATURE CITED

- CHANOCK, R. M., AND A. B. SABIN. 1953. The hemagglutination of St. Louis encephalitis virus. II. Physico-chemical properties and nature of its reaction with erythrocytes. *J. Immunol.* 70:286-301.
- CHANOCK, R. M., AND A. B. SABIN. 1954. The hemagglutination of WEE virus: recovery, properties and use for diagnosis. *J. Immunol.* 73:337-351.
- CLARKE, D. H., AND J. CASALS. 1958. Technique of hemagglutination and hemagglutination inhibition with arboviruses. *Am. J. Trop. Med. Hyg.* 7:561-573.
- COLÓN, J. I., AND J. B. IDOINE. 1964. Factors affecting plaque formation by the infectious ribonucleic acid of the equine encephalitis viruses. *J. Immunol.* 114:61-68.
- COLÓN, J. I., J. B. IDOINE, AND O. M. BRAND. 1963. Mode of action of an inhibitor from agar on growth and hemagglutination of group A arboviruses. *Bacteriol. Proc.*, p. 155.
- HARDY, F. M. 1959. The growth of Venezuelan equine encephalomyelitis virus in various tissue cultures. *Am. J. Hyg.* 70:21-27.
- HEARN, H. J., JR. 1961. Cross-protection between Venezuelan equine encephalomyelitis and eastern equine encephalomyelitis virus. *Proc. Soc. Exptl. Biol. Med.* 107:607-610.
- KLEINSCHMIDT, W. J., J. C. CLINE, AND E. B. MURPHY. 1964. Interferon production induced by statolon. *Proc. Natl. Acad. Sci. U.S.* 63:741-744.
- LIEBHABER, H., AND K. K. TAKEMOTO. 1961. Alteration of plaque morphology of EMC virus with polycations. *Virology* 14:502-504.
- LIEBHABER, H., AND K. K. TAKEMOTO. 1963. The basis for the size differences in plaques produced by variants of EMC virus. *Virology* 20:550-566.
- NAHMIA, A. J., S. KUBRICK, AND P. BERNFELD. 1964. Effect of synthetic and biological anions on herpes simplex virus. *Proc. Soc. Exptl. Biol. Med.* 115:993-996.
- PUCK, T. T., S. J. CIECIURA, AND H. W. FISHER. 1957. Clonal growth in vitro of human cells with fibroblastic morphology. Comparison of growth and genetic characteristics of single epithelioid and fibroblastic cells from a variety of human organs. *J. Exptl. Med.* 106:145-158.
- SCHULZE, I. T. 1964. Reversible inhibition of type 2 dengue virus by agar polysaccharide. *Virology* 23:79-90.
- SCHULZE, I. T., AND R. W. SCHLESINGER. 1963. Inhibition of infectious and hemagglutinating properties of type 2 dengue virus by aqueous agar extracts. *Virology* 19:49-57.
- TAKEMOTO, K. K., AND H. LIEBHABER. 1961. Virus polysaccharide interactions. I. An agar polysaccharide determining plaque formation. *Virology* 14:456-462.
- TAKEMOTO, K. K., AND H. LIEBHABER. 1962. Virus polysaccharide interactions. II. Enhancement of plaque formation and detection of variants of poliovirus with dextran sulfate. *Virology* 17:499-501.
- USHIJIMA, R. N., D. W. HILL, G. H. DOLANA, AND L. P. GEBHARDT. 1962. Plaque mutants of WEE virus. *Virology* 17:356-357.